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Pattern Formation in *Dictyostelium*

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INTRODUCTION

Upon starvation, amoebae of the cellular slime mold *Dictyostelium* undergo a program of development in which individual cells aggregate to form a multicellular slug and, subsequently, a fruiting body consisting of stalk cells and spores (Loomis 1982). Terminal differentiation of stalk cells and spores is preceded by formation of prestalk and prespore cells in the slug stage. *Dictyostelium* has attracted considerable interest in recent years as a model system for studies of cell differentiation. In this paper we focus on the control of prestalk and prespore formation and the organization of the slug stage in which the prestalk/prespore decision occurs.

Pattern of Prestalk and Prespore Cells

Dictyostelium amoebae aggregate chemotactically to form an initially homogeneous multicellular mass. This mass then transforms into an elongated "slug," which migrates over the substratum for varying periods of time depending on environmental conditions (Newell et al. 1969; Schindler and Sussman 1977). Prestalk and prespore cells first appear in the slug stage

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where they form a characteristic spatial pattern: prestalk cells in the anterior third of the slug and prespore cells in the posterior two-thirds. Prestalk and prespore cells differ histologically (Bonner et al. 1955), in buoyant density (Tsang and Bradbury 1981; Ratner and Borth 1983), cell-surface glycoproteins (West and McMahon 1979), metabolic activity (Bonner et al. 1984), expression of specific antigens (Krefft et al. 1983; Tasaka et al. 1983), specific polypeptides (Alton and Brenner 1979; Ratner and Borth 1982; Morrissey et al. 1984), and specific genes (see Chisholm et al., this volume).

Some of the special characteristics of prespores anticipate the subsequent differentiated fate of the cells. For example, spore coat proteins are only synthesized in prespore cells (Devine et al. 1982); prespore cells exhibit a decreased size, an increased bouyant density, and an increased density in electron micrographs (Schapp 1983), all of which anticipate the dehydrated condition of differentiated spores. The characteristics of prestalk cells do not so obviously foreshadow those of the stalk cells, and it has been suggested that prestalk cells most strongly resemble aggregation-stage cells, or that they should be regarded as a transitional stage between aggregation-stage cells and prespores (Schaap 1983, and references cited in Schaap's discussion). Indeed, monoclonal antibodies have been isolated that react with antigens in both prestalk cells and aggregating cells but not with prespores (Tasaka et al. 1983).

Nevertheless prestalk cells can be clearly differentiated from aggregation-phase cells on the basis of their staining with weakly basic dyes such as neutral red and nile blue, apparently due to a specialization of their lysosomes (Sternfeld and David 1981a; Yamamoto and Takeuchi 1983). The prestalk/prespore pattern in slugs can be visualized by neutral red staining; it is particularly apparent in wild-type strains NC-4 and V12, although it is less distinct in the axenic strains Ax2 and Ax3 (in which the two cell types are also harder to distinguish on Percoll gradients [Ratner and Borth 1983]). Prestalk cells also contain a specific isozyme of acid phosphatase (Oohata 1983) that is absent in aggregation-phase cells.

The prespore zone contains, in addition to prespore cells, a population of anteriorlike cells (Sternfeld and David 1982) that resemble prestalk cells in buoyant density and protein synthetic patterns (Ratner and Borth 1983), as well as in size and cell-surface antigens (L.Voet et al., in prep.). The cells are not identical to prestalk cells, however: When prestalk and prespore zones are differentially marked and stirred together, the prestalk and prespore cells sort out from one another; the anteriorlike cells, for the most part, sort to the reconstituted prespore zone (Sternfeld and David 1981a).

Organization of the Slug: The Tip/Body Pattern

A typical slime mold slug has the form of a cylinder with a blunt point, the tip, at the anterior end. When aggregates are initially formed they are not cylindrical but hemispherical, a "default" shape that presumably reflects only

the mutual adhesion of slime mold cells. The first step in normal slug morphogenesis is the appearance of a tip. The subsequent conversion from hemisphere to cylinder often seems to begin immediately adjacent to the tip, progressing thereafter into the rest of the aggregate tissue. When the tip is excised the remainder of the slug relapses into a hemispherical shape, in which it remains until a new tip is formed. When an extra tip is implanted or appears spontaneously in an intact slug, a portion of the host tissue is reorganized into a cylinder posterior to the new tip (the double-tipped slug subsequently splits in two). All of these observations suggest that the tip itself is responsible for the organization of the rest of the cells into a cylindrical form. The tip also appears to be responsible for the elongate form of the slug. In normal development the aggregate begins to elongate shortly after the tip appears; a mutant that makes "stubby" slugs can be rescued by transplantation of wild-type tips (MacWilliams 1984).

The tip is thus a specialized region of the slug, and one can speak of the organization of the slug into tip and body regions. Unlike the prestalk/prespore pattern, the boundaries of the tip cannot be visualized by staining, and it is not clear whether or not one can meaningfully speak of tip and nontip cells. Recent observations suggest that the tip may be definable at the level of cell shape and orientation; it contains a "daisy" pattern, in which a cluster of round cells is surrounded by cells elongated along axes passing through the center of the cluster (J.T. Bonner, pers. comm.). As yet it is not known whether the daisy region is coextensive with the prestalk zone or whether it is specifically associated with the other properties of the tip.

Regeneration of the Prestalk/Prespore and Tip/Body Patterns

When a slug tip is excised, the posterior tissue relapses into a hemispherical form, in which it may remain for up to about 2 hours. In almost all cases, however, the cell mass ultimately regenerates a morphologically recognizable tip (after which it reorganizes into a cylindrical form and may resume migration).

Regulation is also a feature of the prestalk/prespore pattern: When the prestalk zone of the slug is isolated, cell-type conversion ensues with the ultimate result that a prespore zone is reformed. In isolated prespore zones there is a similar, although somewhat more complex, regeneration; a new prestalk zone is formed for the most part by aggregation of anteriorlike cells (Sternfeld and David 1981a). This aggregation seems likely to be oriented by cyclic AMP (cAMP) produced by the prestalk cells themselves (see below). The anteriorlike cells are then replaced by conversion of prespore cells to anteriorlike cells (Sternfeld and David 1982; Takeuchi et al. 1982).

The existence of tip, prestalk, and prespore regeneration shows that cells of all parts of the slug retain the capability to form both of the slug's normal anterior-posterior patterns. Slime mold pattern formation can thus be studied in slugs; this is a substantial advantage from an experimenter's point of view,

since one is not forced to work with a fleeting developmental stage, the normal situation in studies of pattern formation in embryos. It is appealing to assume that the pattern regeneration mechanism in slugs is the same as the mechanism originally responsible for slug formation, but this cannot be proven by studies of slugs alone.

REGULATION OF THE PRESTALK/PRESPORE PATTERN

If one ignores the spatial aspects of the prestalk/prespore pattern, the regeneration of this pattern reduces to a regulation of the proportions of prestalk and prespore cells. Perhaps the simplest way to explain such proportion regulation is via negative feedback. Certain known cell interactions in *Dictyostelium* are well described by a negative-feedback model. Before presenting this evidence, it is helpful to review some formal characteristics of negative-feedback systems.

Negative-feedback Model

An example of a simple negative-feedback mechanism is one in which the proportion of prestalk cells is controlled by an inhibitor of prestalk differentiation that is produced by prestalk cells. In such a system prestalk differentiation (and, hence, the accumulation of inhibitor) continues until the inhibitor concentration reaches a critical level at which further prestalk differentiation is blocked.

The proportion of prestalk cells in such a system will depend on both (1) the sensitivity of the differentiating cells to the inhibitor and (2) the amount of inhibitor produced per prestalk cell. Consider first the consequences of altering the sensitivity. A strain with a high sensitivity to the inhibitor will form a small proportion of prestalk cells, since even low levels of inhibitor (which can be produced by a few prestalk cells) suffice to block further prestalk differentiation. A strain with low inhibitor sensitivity will form a high proportion of prestalk cells, since prestalk differentiation will not be blocked until many prestalk cells have differentiated and relatively high levels of inhibitor have been achieved. Changes in the sensitivity of cells to inhibitor thus lead to simultaneous changes in the equilibrium level of inhibitor and the proportion of prestalk cells that are formed. Specifically, higher sensitivity leads to fewer prestalk cells and lower inhibitor levels; lower sensitivity to inhibitor leads to more prestalk cells and higher inhibitor levels.

Changes in the amount of inhibitor produced per prestalk cell will also lead to changes in the proportion of prestalk cells. For example, if the inhibitor production per prestalk cell is reduced, more prestalk cells are required to produce the equilibrium inhibition level, and the proportion of prestalk cells will be increased. This change is in the same direction as would be produced by a decrease in sensitivity. Unlike a sensitivity decrease, however,

an inhibitor production decrease will not lead to an increase in the equilibrium inhibitor level; the level of inhibitor at equilibrium will either be unchanged (if all cells have the same sensitivity) or will actually be decreased (if there is considerable heterogeneity in sensitivity level).

Coordinate Shifts of Sensitivity, Proportioning, and Inhibitor Level

There are a number of treatments that affect the proportions of prestalk and prespore cells in *Dictyostelium*. The negative-feedback hypothesis can be tested in these cases by looking for coordinate shifts in "prestalk inhibitor level" and "sensitivity to prestalk inhibitor." Both of these formal parameters can be measured in experiments in which one mixes a small fraction of the cells to be tested (donor cells) into a large excess of untreated cells (host cells). Changes in sensitivity are measured by comparing the behavior of the donor and host cells in the chimeric slug; since donor and host cells are exposed to the same inhibition level, differences in behavior imply differences in sensitivity. Changes in inhibitor levels are measured by comparing the behavior of the donor cells in the chimera to their behavior when developing alone.

The coordinate parameter shifts predicted by the negative-feedback model for primary shifts in sensitivity have been identified in three different situations (which follow)

Cells Grown in the Presence and Absence of Glucose

Cells grown in the presence and absence of glucose (G^+ and G^- cells, respectively) differ in several properties including the proportions of prestalk and prespore tissue: G^+ cells form 20% prestalk; G^- cells form 27% prestalk (Forman and Garrod 1977). If the shift in proportions is due to a sensitivity shift, the decreased proportion of prestalk tissue in G^+ cells should be accompanied by an increased sensitivity to prestalk inhibitor and a decreased equilibrium level of prestalk inhibitor.

These predictions have been confirmed in a series of mixing experiments (Leach et al. 1973). When a small proportion of G^+ cells are mixed with an excess of G^- cells, the G^+ cells form almost exclusively spores. The G^- "host" cells form both stalk cells and spores. Since the spore-forming fraction is higher in the G^+ cells than in the G^- cells, one can conclude that the G^+ are more sensitive to prestalk inhibition. Since the fraction of G^+ cells that form stalk is higher in a pure G^+ slug than it is in the G^- host, one can conclude that the level of prestalk inhibition in the pure G^+ host is lower than in the G^- slug.

Proportioning Mutants

More recently, mutants have been isolated in which the proportions of prestalk and prespore cells are altered (MacWilliams 1982). Two complementa-

tion groups of "short prestalk" mutants are known, in which the proportion of prestalk cells is reduced from about 15% (in the wild type) to 2–5%. In mixtures with wild-type cells the mutants preferentially form prespore cells, indicating an increased sensitivity to "prestalk inhibitor." When small numbers of wild-type cells are added to short prestalk slugs, virtually all of the wild-type cells form prestalk, indicating a lower inhibition level in the mutant slugs (A. Blaschke et al., in prep.). Thus, the mutants show both the changes predicted by the negative-feedback model for an increase in prestalk inhibitor sensitivity.

Two complementation groups of "long prestalk" mutants have been identified in which the proportion of prestalk cells is increased (from ~15% to 40%). In mixtures with wild-type cells, both mutants preferentially make prestalk cells, indicating a decreased sensitivity to prestalk inhibitor. When small numbers of mutant cells are mixed with an excess of wild type, they form exclusively prestalk and anteriorlike cells (A. Blaschke et al., in prep.). Thus, it appears that the prestalk inhibition level in mutant slugs is higher than in wild type, as expected from a decrease in sensitivity to prestalk inhibition.

Synchronized Cell Populations

It has recently been discovered that synchronized cell populations exhibit large variations in prestalk/prespore proportions, depending on the cell-cycle phase at which development is initiated. Immediately postmitotic cells form slugs with about 45% prestalk tissue, whereas cells in late G_2 phase and stationary-phase cells form slugs with about 10% prestalk tissue (Weijer et al. 1984). Postmitotic cells thus resemble the long prestalk mutants discussed above.

When postmitotic cells are mixed with cells of random cell-cycle position, they preferentially form prestalk cells, indicating that their sensitivity to prestalk inhibition is lower than that of random-cycle cells. When a small fraction of random-cycle cells are mixed with an excess of immediately postmitotic cells, the random-position cells form, almost exclusively, prespore cells, although they form cells of both types when differentiating alone. This indicates that the level of prestalk inhibitor is increased in slugs developing from postmitotic cells, as expected if their sensitivity to prestalk inhibition is decreased. The behavior of synchronized cells in mixtures thus suggests that the sensitivity to prestalk inhibitor varies with the cell-cycle position at which development is initiated.

Observations on glucose-grown cells, proportioning mutants, and synchronized cells thus all show the coordinate shifts in proportioning, sensitivity to prestalk inhibition, and prestalk inhibition level, which are predicted by a simple negative-feedback model of prestalk/prespore proportioning. This supports the idea that negative feedback is a fundamental component of the mechanism regulating prestalk and prespore proportions. It should be clear

that the specific features of the negative-feedback model discussed here are arbitrary; negative feedback could also be achieved, e.g., by a "prespore inhibitor"—a substance produced by prespore cells that is necessary for prestalk formation. In this case, just as in the case of a prestalk inhibitor, coordinate changes in proportioning, sensitivity, and signal level are expected.

Possible Negative-feedback Regulatory Substances

Various experiments suggest the possible involvement of at least three different factors in the partition of the slug into prestalk and prespore cells. In all cases, the available data are consistent with the idea of negative-feedback regulation of cell-type proportions, although the factors do not necessarily have the specific function of the prestalk inhibitor discussed above.

Differentiation-inducing Factor

When amoebae of the strain V12/M2 are plated at high density on agar containing millimolar cAMP and are prevented from aggregating by an overlay of cellophane, the cells differentiate quantitatively into stalk cells. Low-density cells fail to differentiate but will do so if provided with a factor released by high-density cells (Town and Stanford 1979). This factor, which has been purified but not identified, is referred to as differentiation-inducing factor (DIF).

DIF shows clear effects on cell-type proportioning in an in vitro system employing "sporogenous" mutants. In these mutants the normal cell-contact requirement for differentiation appears to be bypassed; both prestalk and prespore cells continue their normal differentiation (into stalk cells and spores) when plated at low density on cAMP agar (Tsang and Bradbury 1981). Addition of DIF to the medium shifts cells from the spore to the stalk pathway in a concentration-dependent fashion; 100% stalk formation can be obtained (Kay and Jermyn 1983).

The idea that DIF plays a role in the normal control of prestalk/prespore proportioning is supported by the observation (Brookman et al. 1982) that DIF production begins between aggregation and slug formation, the stage at which prestalk and prespore differentiation is first demonstrable. Mutants unable to produce DIF (Kopachik et al. 1983) are blocked at this developmental stage; in particular, they fail to form tips. It seems possible that the failure to form tips results from an inability to form prestalk cells (see below). The phenotype of the mutants is thus consistent with the idea that DIF is necessary for prestalk cell formation.

Studies on the distribution of DIF in slugs suggest that the concentration of the factor is actually higher in the rear of the slug than in the prestalk zone (J. Brookman, pers. comm.). This is consistent with the hypothesis (see Meinhardt 1983) that DIF is produced by prespore cells and broken down in the prestalk zone. If this is the case, one expects that an excess of prespore

cells would lead to an excess of DIF, inducing conversion of prespore to prestalk. Conversely, if prestalk cells were in excess, the DIF concentration would fall, bringing about conversion in the opposite direction. This is clearly recognizable as negative-feedback regulation of cell-type proportions, in which DIF plays the role of a prespore inhibitor, rather than a prestalk inhibitor as discussed above.

Ammonia

Ammonia is secreted in large quantities by differentiating cells; it is present at about 10 mM in typical suspension cultures of developing cells (10^6 cells/ml, incubated 1 day). The first suggestion that ammonia might play a role in controlling cell differentiation came from experiments showing that it was required at a level of 3–5 mM for spore differentiation in aggregates of cells developing in suspension (Sternfeld and David 1979).

More recent experiments have shown that ammonia can affect the cell-type proportions in the single-cell differentiation system developed for studies on DIF. Concentrations of ammonia in the range of 3–5 mM convert cells from stalk to spore differentiation (Gross et al. 1983). This concentration range is similar to that affecting spore differentiation in submerged aggregates.

Further evidence that ammonia is involved in cell-type proportioning comes from the observation that three short prestalk mutants can be shown to be ammonia-hypersensitive in an assay independent of proportioning (the assay is based on suppression of fruiting-body formation [see Schindler and Sussman 1977]). Two of the mutants, KY3 (Yanigasawa et al. 1967) and NP429 (Newell and Ross 1982), were originally isolated due to their failure to fruit and were subsequently found to be defective in proportioning; the third, HS2 (MacWilliams 1982), was initially identified as a proportioning mutant.

If ammonia were to function as a negative-feedback substance, one would have to imagine that it is produced primarily by prestalk cells and that it inhibits prestalk differentiation. "Ammonia-sensitive" mutants would then form a reduced prestalk zone and would produce low levels of ammonia. There is at present no evidence that ammonia is primarily produced by prestalk cells or that ammonia-sensitive mutants produce ammonia at reduced levels.

cAMP

Early proposals that cAMP might be specifically involved in stalk differentiation have largely given way to the idea that this substance is not pathway-specific, being essential for both prestalk and prespore differentiation (Gross et al. 1981; see also Chisholm et al., this volume). There is, however, some evidence that cAMP may specifically stabilize prespore cells. When prestalk and prespore cells are separated on density gradients and allowed to form aggregates, cell-type conversion normally occurs in both sorts of aggregates to restore the original cell-type proportions. Physiological concentrations

$(10^{-6}-10^{-5} \text{ M})$ of cAMP in the external medium block cell-type conversion in prespore aggregates and stimulate it in prestalk aggregates. These observations are consistent with the idea that cAMP specifically stabilizes prespore cells (Weijer and Durston 1984).

Further evidence for the role of cAMP as a pathway-specific morphogen comes from studies of a mutant capable of differentiation in the unaggregated state. Below 10^{-6} M cAMP, this mutant forms only stalk cells; above 10^{-5} M cAMP, only spores are formed (Ishida 1980).

These results, together with others suggesting that cAMP is preferentially produced by prestalk cells (Pan et al. 1974; Brenner 1977; Town and Stanford 1977), are consistent with the idea that cAMP is a prestalk inhibitor.

CELL SORTING IN THE FORMATION OF THE PRESTALK/PRESPORE PATTERN

Sorting of slime mold cells can be demonstrated in a variety of situations. When, as previously mentioned, the prestalk and prespore zones are differentially marked and stirred together, the two cell types sort out from one another; in this case the prestalk/prespore pattern is *reconstituted* by cell sorting (Sternfeld and David 1981a; Siu et al. 1983). *Regeneration* of the prestalk zone in prespore fragments is also largely a matter of cell sorting, in this case sorting of anteriorlike cells from prespore cells (Sternfeld and David 1981a). It is therefore clear that *differentiated* slug cells can form a pattern by sorting.

Sorting, moreover, can also be observed in mixtures of slime mold cells that were not differentiated at the time of mixing (see preceding section); thus, when vegetative G^+ and G^- cells are mixed, they largely become separated at the slug stage. The same applies to mixtures of proportioning mutants and wild-type cells. The recent studies on sorting according to cell-cycle phase strongly suggest that sorting occurs even in natural (i.e., unmixed) populations of developing *Dictyostelium* cells.

Mechanism of Cell Sorting

Cell sorting in slime molds has features suggestive of both differential chemotactic and differential adhesive mechanisms. Suggestive of chemotaxis is the observation that the sorting of prestalk cells (within a mixture of prestalk and prespore) can be oriented by concentration gradient cAMP (Matsukuma and Durston 1979; Sternfeld and David 1981a). Oxygen gradients are also known to orient sorting (Sternfeld and David 1981b), possibly by induction of a cAMP gradient. Since slug cells are known to respond chemotactically to cAMP (Maeda 1977), it is quite easy to imagine that prestalk/prespore sorting is mediated by differential chemotaxis toward cAMP. However, no difference in chemotactic ability between prestalk and prespore cells has as yet been demonstrated.

Suggestive of adhesive sorting is the observation that prestalk and prespore cells differ in adhesiveness (Maeda and Takeuchi 1969; Yabuno 1971; Siu et al. 1983; C. Weijer, in prep.). Furthermore, Siu et al. (1983) have shown that Fab fragments directed against GP150, a molecule implicated in slug cell adhesion, block the sorting out of prestalk and prespore cells. On the basis of this observation, these investigators proposed that prestalk and prespore cells sort out via a simple differential adhesive mechanism (Steinberg 1963) driven by differences in cell-surface GP150. However, such a mechanism, in which sorting is brought about by chance collisions among randomly moving cells, can only give rise to simple inside-outside sorting patterns in aggregates of cells. It cannot explain the observed orientation of prestalk/prespore sorting patterns under the influence of imposed external gradients of cAMP or oxygen (Sternfeld and David 1981a,b).

At least two simple models can be envisioned that account for both the importance of GP150 in sorting and the appearance that sorting is chemotactic. One possibility is to assume that sorting is basically chemotactic but that prestalk and prespore cells are only *differentially* chemotactic due to a difference in adhesiveness; one might imagine, e.g., that adhesive differences create different degrees of "drag" on cells moving within the slug (C. Weijer et al., in prep.). Suppression of the adhesive differences would lead to an abolition of sorting.

Alternatively, one might imagine the possibility of "oriented adhesive sorting." It has been shown (C. Weijer et al., in prep.) that cAMP continuously modulates the adhesiveness of slug cells. A cAMP gradient could thus be translated, within an aggregate, into a gradient of cell adhesiveness. In such a gradient, individual cells are expected to seek their own levels, i.e., move toward positions where the average cell adhesiveness equals their own adhesiveness (MacWilliams 1984). If prestalk and prespore cells differ in adhesiveness, they should move differentially within an imposed adhesive gradient, giving rise to the appearance of differential chemotaxis toward cAMP. In such an oriented adhesive sorting model one might imagine that GP150 is responsible either for the overall adhesive gradient or for the adhesive difference between prestalk and prespore cells; suppression of either one of these adhesive differences would abolish sorting.

Role of Cell Sorting and Position-dependent Cell Differentiation in the Formation of the Prestalk/Prespore Pattern

Cell-differentiation patterns in higher animals and plants are widely considered to arise via position-dependent cell differentiation, presumably brought about by position-dependent signals (Wolpert 1971). It is an open and much-disputed question whether position-dependent cell differentiation contributes to the formation of the prestalk/prespore pattern in slime molds. An alternative possibility is that pattern in slime mold slugs is created entirely by cell

sorting. Although it seems that one should be able to distinguish position-dependent differentiation from sorting with simple, formal experiments, so far a clean distinction has not been made. It is clear that sorting occurs, but it has proven difficult to determine whether sorting causally *precedes* or *follows* differentiation into prestalk and prespore cells.

Two hypotheses seem equally reasonable. In one, in which the prestalk/ prespore pattern is formed by sorting, cells are randomly distributed in the aggregate at the time that cell differentiation begins and sort out subsequently due to properties they acquire as a consequence of cell differentiation. In a second hypothesis, in which the pattern is formed by position-dependent cell differentiation, cells with high and low sensitivity to prestalk inhibition establish through sorting a gradient of sensitivity, such that the cells with lowest sensitivity occupy the portion of the slug where the prestalk zone will ultimately appear. Since sensitivity to prestalk inhibition appears to be a continuously variable parameter, a sensitivity gradient does not yet contain two distinct cell differentiation zones. A subsequent position-dependent mechanism then "draws a boundary" in the slug such that the anterior cells become prestalk and the posterior cells become prespore.

There are several facts that initially seem to support one or the other of these hypotheses but that do not in fact permit one to make much progress against a stubborn advocate of the opposing point of view.

Localized Appearance of Differentiation Markers.   In principle, by following the appearance of an early differentiation marker, it should be possible to determine whether differentiation occurs in single randomly distributed cells or in a coherent region. Several experiments of this sort have been done using polyclonal (Tasaka and Takeuchi 1981) and monoclonal (Krefft et al. 1984) antibodies to prespore cells. The results indicate clearly that, under normal developmental conditions, these prespore markers first appear at the rear of the prespore zone and then "spread" toward the prestalk/prespore boundary. This is not a strong argument for position-dependent differentiation, however, since the prespore zone might "grow" by accretion of cells that had reached their decision to become prespore while spatially dispersed and had migrated to the prespore zone shortly afterwards.

Random Appearance of Differentiation Markers.   There are also a number of examples in which signs of differentiation appear in randomly dispersed cells. (1) In cell aggregates developing in suspension, a pepper-and-salt pattern of cells expressing or not expressing prespore antigens is observed (Tasaka and Takeuchi 1981). (2) In *Dictyostelium minutum* prestalk and prespore cells are reported to be formed although there are no distinct prestalk and prespore zones (L. Schaap, unpubl.). (3) In *D. discoideum*, cells showing dehydration of the cytoplasm can be observed in randomly dispersed cells in aggregation streams before slug formation has occurred (Schaap 1983). (4) Spatially disorganized differentiation of stalk cells and spore occurs in the

monolayer system, in which cells plated at low density differentiate without appreciable cell contact (Kay and Jermyn 1983). (5) New anteriorlike cells (identified by vital staining) that differentiate during regeneration of slug posteriors appear in a spatially disorganized fashion (Takeuchi et al. 1982). (6) In some mutants blocked in tip formation, cells that are either prestalk or anteriorlike (identified by vital staining) appear in a random distribution (S. Ishida, pers. comm.). In all of these cases, one has at most shown that pre-stalk and prespore cells *can* differentiate without spatial cues; this falls short of proving that spatial cues do not exist in normal development.

Existence of Sorting Gradients. Sorting gradients can be demonstrated within the prestalk and prespore zones; thus, anterior prestalk cells sort from posterior prestalk cells. In mixtures of proportioning mutants and wild-type cells the relative frequency of the two cell types depends upon position in the zones (see MacWilliams 1984). These observations can be explained by the idea that sorting primarily produces a gradient within slugs, rather than a two-zone pattern, and thus appear to support the idea that sorting precedes differentiation. They are also consistent, however, with the concept that sort-ing causally follows cell differentiation: If a prestalk cell is a cell that sorts to the anterior zone of a slug, a cell that is "more prestalk" than others could preferentially sort to the anterior portion of this zone.

In summary, it appears to us that distinguishing between sorting and posi-tional hypotheses may ultimately only be possible at the molecular level. Presumably differential sorting (within, as well as between, zones) ulti-mately reflects differential expression of certain specific genes. If these genes were found to be regulated by the same factors that regulate the expression of prestalk- or prespore-specific genes, this would speak for the sorting-out model of pattern formation. If, on the other hand, the sorting genes were differentially regulated in nondifferentiated cells, this would support the idea that sorting precedes the formation of the two cell types and thus precedes pattern formation. This would be consistent with the idea that differentiation is brought about by position-dependent signals.

REGULATION OF THE TIP/BODY PATTERN

Negative Feedback from the Tip

When small groups of slug cells are introduced into a second slug, they may form tips (Durston 1976; MacWilliams 1982). Transplantation experiments suggest that tip formation is under negative-feedback control. Thus, (1) the slug tip exerts an inhibitory effect on tip formation; removing the tip of the host slug substantially increases the frequency of such tip formation, as does increasing the distance between host tip and transplantation site. (2) The cells of the slug tip itself appear to be resistant to the inhibition; transplanted tip cells form tips readily, whereas transplanted body cells do not. Such a dif-

ference in resistance is expected within a negative-feedback system; otherwise the tip/body pattern would not be stable.

Relation to the Prestalk/Prespore Pattern

The appearance of negative feedback in the tip/body system reminds us of the system of prestalk and prespore cells. Further experiments suggest that the two systems are indeed related. Transplantation studies on slugs show that the region of high resistance to tip inhibition is coextensive with the prestalk zone; this is true in both wild-type slugs and in mutants in which the position of the prestalk/prespore boundary is shifted (Durston and Vork 1977; MacWilliams 1982). Resistance of prestalk cells to tip inhibition can also be inferred in experiments in which these cells (within an aggregate that also contains prespores) are caused to cluster about an electrode emitting cAMP. When the electrode is withdrawn, tip formation may occur (Matsukuma and Durston 1979).

These findings can be taken as suggesting that the tip and the prestalk zone are identical. Two further transplantation results, however, indicate that this is not the case. (1) Tip formation can be induced by transplants of anterior prestalk tissue into the posterior part of the prestalk zone (H.K. MacWilliams, unpubl.). A single prestalk zone is not necessarily a single tip; rather, the tip is a substructure of the prestalk zone. (2) If all prestalk cells were tip cells, negative-feedback models lead one to expect that all prestalk cells produce tip inhibition; the tip inhibition and prestalk inhibition would be the same. Attempts to confirm this fail; removing part, or even most, of the prestalk zone has little effect on the tip inhibition level (MacWilliams 1982 and unpubl.)

Studies of the transplantation properties of proportioning mutants support the idea of a limited connection between prestalk/prespore and tip/body patterning. Thus, in the short prestalk mutants HS2 and NP429, in which the sensitivity to prestalk inhibition is increased and the prestalk inhibition level is decreased, one finds a parallel increase in the sensitivity to tip inhibition and decrease in tip inhibition level (MacWilliams 1982). Since in both systems the shift in inhibition level may follow from the shift in sensitivity, the parallel behavior of the tip/body and prestalk/prespore systems is sufficiently explained by the assumption that the mutations coordinately affect tip and prestalk inhibition sensitivity. It is not necessary to assume that the mechanism of inhibition in the two systems is the same.

The idea that the tip/body and prestalk/prespore systems have separate inhibition mechanisms is consistent with the idea that the two systems have significantly different functions. In a recent model (Meinhardt 1983) the prestalk inhibition system is designed for exact prestalk/prespore proportioning, whereas the tip inhibition system is optimized for developmental speed. One could also imagine that the tip inhibition system is specialized

for the control of the morphogenetic movements of later development. If the tip/body pattern and the prestalk/prespore pattern are regulated by different mechanisms, the coupling between the two systems (suggested by the transplantation properties of proportioning mutants) may be interpreted as a means of assuring that the patterns have a consistent mutual orientation.

Waves and the Tip Inhibition

It is conceivable that the tip inhibition is mediated by cAMP waves similar to those that dominate slime mold aggregation. Aggregation waves arise at "pacemakers"—cell groups that periodically emit pulses of cAMP—and are "relayed" outward from these centers because the other cells are "excitable"—pulses of cAMP stimulate them to emit such pulses themselves. The pulse emitted by one cell stimulates its neighbor, which in turn emits a pulse, and so forth. The cells in pacemakers may not be qualitatively different from other slime mold cells; it is possible that all cells oscillate but that pacemakers oscillate faster and "entrain" other cells by stimulating them to emit their cAMP pulses ahead of the time when they would have done so spontaneously.

Oscillatory cAMP release can be measured in suspensions of aggregation-competent slime mold cells (see Chisholm et al., this volume). C.J. Weijer (in prep.) has measured such oscillations in cells of different cell-cycle positions; the postmitotic cells that are fated to become prestalk cells oscillate faster than those that will become prespore. If these differences are maintained in the slug, it is reasonable to expect that the cells of the slug front oscillate spontaneously and act as a pacemaker for the rest of the slug. The nontip cells would then be "inhibited" in the sense that they would be prevented from initiating cAMP waves on their own.

Can the phenomenon of tip inhibition be explained by this concept of entrainment? Imagine a transplant whose natural frequency is as high or higher than the frequency of the host tip. This transplant would resist entrainment, it would persist in oscillating at its natural frequency, and it would initiate waves that would spread into the host. A wave cancellation front would be formed at some point between the host and the transplant pacemakers. Presuming that slug cells in general migrate toward the source of the entraining waves, the slug body would be partitioned at the cancellation front between the host and transplant pacemakers. In transplantation experiments one in fact observes partitioning between host and transplant tips; when no tip is formed the transplant is soon unrecognizable in the body of the host. The concept of a tip as a pacemaker allows one to explain at least its basic morphogenetic activity.

In a wave model, the level of the tip inhibition would be identified with the pacemaker frequency of the host, whereas the level of resistance to tip inhi-

bition would be identified with the spontaneous oscillation frequency of the transplant. Simple transplantation experiments (Durston 1976; MacWilliams 1982) demonstrate a gradient along the slug in resistance to tip inhibition; according to wave models, this implies that a *gradient in natural frequency* exists in the slug. The wave model predicts that this gradient should also be detectable in transplantation experiments of quite different design, in which one cuts tissue from the front of the slug and measures the *tip inhibition* (not the resistance to it) in the remaining piece. When tissue is removed from the front of the slug, the oscillation frequency in the remaining piece should drop to the natural frequency of the cells at its anterior end. This frequency will then determine the inhibition level in the piece. By cutting at various positions and measuring inhibition level, one should be able to measure the natural frequency gradient in the slug. When this experiment is performed, it indeed gives a curve identical to the measured gradient in resistance to tip inhibition (MacWilliams 1982). This supports the idea that the tip inhibition is mediated by waves.

Wavelike phenomena can in fact be demonstrated directly in slugs. Time-lapse studies of prestalk cells introduced into the prestalk zone show that these cells migrate forward with an oscillating velocity; neighboring cells oscillate in phase, as if under the influence of a supramolecular, oscillating stimulus. When the tip is removed from the slug, the oscillation frequency drops (Durston and Vork 1979). The wave concept is further supported by the fact that the migrating prestalk cells move along spiral paths (Durston et al. 1979); spiral motion is often seen in slime mold aggregation and is easily explained as the consequence of a rotating wave.

Dependence of Inhibition on Tip Size

A characteristic of wave models is that the effectiveness of inhibition need not depend on tip size; small tips should be able to inhibit a given cell mass as effectively as large ones. In a study of the inhibition produced by the tips of large and small slugs, Kopachik (1982) found that the inhibition depends somewhat on tip size. Over a 100-fold range of mass of tips transplanted to a constant-sized tipless aggregate, there was a change from 25% to 80% inhibition of secondary tip formation. In a similar assay system there is a massive difference in the response to equal-sized tips of wild-type and short prestalked strains (H.K. MacWilliams, in prep.), suggesting that factors other than tip size are most important in determining the tip inhibition level.

In the wave model, a *moderate* dependence of tip natural frequency on slug size would not be surprising; slow pacemakers would be expected, on the average, to have captured fewer cells during the pacemaker competition of aggregation. Small slugs indeed show a moderately reduced inhibition level; cells from their tips are moderately more sensitive to tip inhibition than cells from the tips of large slugs (H.K. MacWilliams, in prep.).

Tip Inhibition Gradient

One transplantation phenomenon appears at first glance to be inconsistent with wave models. This is the fact that the tip inhibition falls off in intensity at increasing distances from the tip. One's naive expectation is that the frequency of a wave should be the same in all parts of the slug. If the slug contains a natural frequency gradient, however, it is possible that the cells of the slug rear simply cannot be driven at the frequency of the slug tip. In this case, occasional wave fronts would simply cease to propagate at some anterior-posterior position, giving rise to a genuine frequency gradient.

SUMMARY: THE ORIGIN OF SPATIAL ORGANIZATION IN SLIME MOLD DEVELOPMENT

There appear to be two relatively independent regulatory systems in slugs: one regulating prestalk/prespore proportions and the other regulating tip/body organization. Since it has many features of the chemotactic aggregation system, it seems likely that the major function of the tip/body system is to control morphogenetic movements: those involved in slug migration and those involved in culmination. The major function of the prestalk/prespore system is to create the appropriate proportions of the two cell types; the tip/body system then uses these cells to create fruiting bodies (either "stalky" ones or "spory" ones, depending on the proportions of cells at its disposal). The prestalk/prespore and tip/body systems appear to be coupled in inhibitor sensitivity; this assures that they will have a consistent mutual orientation.

In the formation of either the prestalk/prespore or tip/body pattern, the developing slime mold aggregate faces what might be called the general problem of biological pattern formation: to create restricted, spatially coherent regions of cell differentiation in a tissue in which all cells are capable of the differentiation in question. In theoretical models of pattern formation this is often accomplished by a combination of positive and negative feedback. Negative feedback with a long spatial range restricts the differentiation to a subset of the cells. Short-range autocatalysis accounts for the differentiation of cells in clusters.

In prestalk/prespore patterning, negative feedback appears to exist, and several candidates for the negative-feedback substance have been identified. There is no evidence, however, that either cell type produces a diffusible activator that stimulates other cells to differentiate in the same fashion. The available evidence in fact speaks against this. Thus, when small groups of prestalk cells are transplanted into the prespore zone, they do not characteristically become prespore cells; instead they either form a tip and thereby a new prestalk zone or migrate to the prestalk zone of the host. Similarly, prespore cells transplanted into the prestalk zone can be quantitatively recovered from the prespore zone several hours later (J. Morrissey, in prep.).

A positive-feedback loop of a somewhat different sort is readily identified,

however, in the relationship between the prestalk cells and the tip. Thus, (1) prestalk cells in slugs are attracted to the tip of the slug, and (2) prestalk cells have a higher resistance to tip inhibition than do other slug cells. A tip thus provides a means by which prestalk cells can attract other prestalk cells. Tip formation could thus be a self-reinforcing process; by attracting prestalk cells, an incipient, weak tip region could develop into a strong tip. This means, in turn, that tip formation could be oriented by a very weak initial stimulus.

Let us consider how this mechanism might work in normal slime mold development. Assume that aggregation has produced a mass of cells resting on a moist substrate. As a first step, one might imagine that diffusion of oxygen from above and diffusion of cAMP into the substrate below could create weak gradients of both of these substances across the aggregate; in each case, the maximum would be at the pole of the aggregate most removed from the substrate. Concurrently, the prestalk/prespore negative-feedback mechanism might cause the formation of randomly distributed prestalk cells. Since both cAMP and oxygen appear to attract prestalk cells, one would then expect the newly produced prestalk cells to become enriched in the upper portion of the aggregate. Since it seems likely that prestalk cells produce larger amounts of cAMP than other cells, this initial enrichment of prestalk cells in the apical portion of the aggregate should reinforce the existing cAMP concentration gradient, leading in turn to further apical enrichment of prestalk cells.

Accumulation of prestalk cells in the apical portion should also create a region of increased natural frequency, and at some point one would expect a pacemaker to be formed at the apical pole. Propagating waves might then destroy the initial cAMP concentration gradient, but the natural frequency gradient in the slug would be maintained by the continued attraction of prestalk cells toward the wave source. Slug formation could then occur under the control of the pacemaker. Cells in the prestalk and prespore zones of the slug would presumably experience different microenvironments, which could contribute to the further differentiation of the prestalk and prespore cells and their ultimate differentiation into stalk cells and spores.

In this outline we assumed that both static and oscillatory signals play a role in the aggregation of prestalk cells. The relative roles that the two sorts of signals play is unimportant in the overall view. Thus, adequate sorting could be achieved on the basis of static concentration gradients alone. Alternatively, if oxygen and cAMP act directly on the natural oscillation frequency of the aggregate's cells, a natural frequency gradient could be created before there is appreciable sorting; a pacemaker could then originate early and might be the sole agent responsible for orienting the aggregation of prestalk cells.

It is similarly immaterial in the overall scheme whether sorting follows prestalk/prespore differentiation, as assumed here, or precedes it. If sorting precedes differentiation, however, one needs to assume that the (not yet pre-

stalk) cells that sort to the apex of the aggregate either have a higher natural oscillation frequency than other cells or that they produce larger amounts of cAMP; either of these assumptions suffices to ensure that sorting will be a self-amplifying process.

The fact that is clearest at this point is that generation of spatial order in cellular slime molds need not be regarded as mysterious. Mechanisms have been characterized that suffice to explain the known facts about the prestalk/ prespore and tip/body patterns. The challenge to researchers in the future will be to determine which of the possible mechanisms play what roles in actual normal slime mold development.

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